UNIVERSITY OF GONDAR

COLLEGE OF MEDICINE AND HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY

EVALUATION OF THE EFFECTS OF THE ROOT EXTRACT OF *ASPARAGUS AFRICANUS LAM.* ON GLUCOSE HANDLING IN NORMAL, GLUCOSE LOADED AND STREPTOZOCIN INDUCED DIABETIC RODENTS

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As members of examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Kefyalew Ayalew entitled as Investigation on: **Evaluation of the Effects of root Extract of *Asparagus africanus Lam.* on Glucose Handling in Normal, Glucose Loaded and Streptozocin Induced Diabetic Rodents** and I recommended that it will be accepted as fulfilling the thesis requirement for the degree of Master of Science in pharmacology.

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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the University of Gondar through the Department Pharmacology of the candidate's major department. I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling thesis requirement.

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List of abbreviations and acronyms

A1C:	Glycated hemoglobin
ANOVA:	One-way analysis of variance
BGL:	Blood Glucose Level
DM**:**	Diabetes Mellitus
FPG:	Fasting plasma glucose
G:	Gram
GDM:	Gestational Diabetes Mellitus
GL5:	Glibenclamide 5mg
GSH:	glutathione
IDF:	International Diabetes Federation
IGT:	Impaired Glucose Tolerance
LD50:	Lethal Dose 50
OECD:	Organization for Economic Co-operation and Development
2-h PG:	Two hours Plasma Glucose
ROS:	Reactive oxygen species
SEM:	Standard Error of the Mean
STZ:	Streptozocin
WHO:	World Health Organization

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Abstract

Background: Diabetes mellitus (DM) is a major metabolic disorder characterized by chronic hyperglycemia; with disturbances of carbohydrate, protein and fat metabolism resulting from defects in insulin secretion, insulin action or both. Despite the introduction of hypoglycemic agents, diabetes and related complications continue to be a major health problem in the 21st century. In addition, many oral hypoglycemic agents have got major side effects and cannot be used during pregnancy. Therefore, searching new antidiabetic agents of less toxic plant origin are continued to be an area of active research as WHO also recommends. Therefore the main objective of this study was to investigate the oral antidiabetic activity of crude extract of root of *Asparagus africanus Lam* in Rodent models.

Materials and method: In all models, rodents were divided in to five groups each comprising of six animals. In hypoglycemic model healthy fasted mice and for antidiabetic studies Streptozocin 150 mg/kg induced diabetic mice, whereas for oral glucose tolerance test (OTT) rats were used. For group I distilled water was give as negative control, group II received 5mg/kg glibenclamide standard (GL5); groups III-V the extract of *A. africanus* 100 (AA100), 200 (AA200) and 400 (AA400) mg/kg/day, were given respectively. Blood samples were collected at different time points to determine blood glucose levels (BGL). Data were analyzed using one way ANOVA followed by Dunnet's post hoc test and $p<0.05$ was considered as statistically significant.

Results: In normal fasted mice, extract 200 mg/kg/day induced hypoglycemia starting from the 2nd h ($p<0.05$) but AA100 mg/kg/day and AA400 mg/kg/day failed to produce hypoglycemia at all time points. In Streptozocin induced diabetic mice AA200 mg/kg/day and AA400 mg/kg/day reduced BGL significantly since the 2nd h but at the 4th h lowered BGL very significantly ($p< 0.001$ in both cases). However, AA100 mg/kg/day revealed effect only at the 4th h ($p<0.05$) in diabetic mice. In OGTT, AA200 and GL5 ($p<0.001$) achieved effect since 60 min indicating the oral glucose load improving activity of the extract. It is worth nothing finding that AA200mg is competing with the standard in all models. Acute oral toxicity test revealed the extract was non toxic up to 5000 mg/kg. Phytochemical screening demonstrated presence of bioactive molecules flavonoids, alkaloids, phenolic and others which ameliorates diabetes and its complications.

Conclusion: The results of this study indicate *A. africanus* has potential antidiabetic actions, particularly at the dose of 200 mg/kg in experimental rodents supporting the traditional claim.

Keywords: *Asparagus africanus*, Antidiabetic activity, Diabetes mellitus, Streptozocin.

1. INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a group of major metabolic disorder of multiple etiologies characterized by chronic hyperglycemia; with disturbances of carbohydrate, protein, and fat metabolism, resulting from defects in insulin secretion, insulin action, or both (1). A number of studies also showed that impaired metabolism is usually associated with oxidative stress, mediated mainly by hyperglycemia induced generation of excessive free radicals through lipid peroxidation, alteration of the activity of several proteins, aggravated by a drastic drop in antioxidant immune mechanisms. This imbalance generally lead to oxidative stress which is the main factor associated with the severity and death in diabetes patients (2, 3).

The acute complication of DM is ketoacidosis, dehydration electrolyte imbalance and others. Its long-term complications are retinopathy with potential loss of vision; nephropathy resulting in renal failure; peripheral neuropathy with risk of foot ulcers, amputations and patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. The risk of micro vascular and neuropathic complications are related to both duration and the severity of hyperglycemia (1, 4)

According to the latest International Diabetes Federation (IDF) estimation in 2013, the numbers of adults living with DM were about 382 million, representing 8.3% of the global adult population, which was projected to rise to 592 million people or 10.1% of adults by 2035 (4, 5)

Currently there is a steady increase in the number of younger individuals with type II diabetes; mainly due to an increase in prevalence of obesity, risk factor for type II diabetes children (6).

Globally DM is considered as the fourth or fifth leading cause of death in most industrialized countries accounting for about 5.1 million deaths in 2013. Every six seconds a person dies from diabetes. This shows diabetes is undoubtedly one of the most challenging health problems of the 21st century. As the prevalence of type II diabetes grows in low and middle income countries, so too does the impact of these cost in both human and economic terms (4). In Africa, currently DM is rapidly spreading, because of rapid uncontrolled urbanization and westernization of life-style and dietary habits with varying prevalence across continent (6).

According to the extrapolated data, the prevalence of DM in Ethiopia was 4.9% in 2013 (5). A study done in 2014 showed the prevalence of diabetes mellitus among adults aged 35 years and above was 5.1% and 2.1% among people who lived in urban and rural respectively (7).

Globally health expenditure spending to treat diabetes and manage its complications in 2013 was USD 548 billion and it will be speculated to exceed USD 627 billion by 2035 (4).

Although to this extent DM is the major public health problem about 80% of the world population, especially resource limited countries benefited from the herbal medicine and greater than 50% of all drugs, in clinical use are herbal products or their derivatives (8). Therefore searching of novel antidiabetic plant origin is one of the active research areas of researchers.

1.1.1 Types of Diabetes mellitus

DM is classified on the basis of the pathogenic process that leads to hyperglycemia, in to two broad categories as type I and type II (9-11). Type I DM majorly results from autoimmune beta cell destruction of the pancreas, which leads to complete or near-total insulin deficiency. Some develop insulin deficiency by unknown mechanism. It is most commonly develops before the age of 30 years but can develop at any age, since an autoimmune beta cell destructive process can occurs at any stage of life (6). Type II DM is characterized by impaired insulin secretion, insulin resistance, excessive hepatic glucose production, and abnormal fat metabolism (1). Obesity, particularly visceral or central is very common in type II DM. Type II DM has a strong genetic component. The concordance of type II DM in identical twins is between 70 and 90%. Individuals with a parent with type II DM have an increased risk of diabetes; if both parents have type II DM, the risk approaches 40. There are Gestational Diabetes Mellitus (GDM) and others specific types of DM (1, 9)

1.1.2 Risk factors of diabetes mellitus

The modifiable risk factors which are associated with type of diabetes include increase in sedentary lifestyle, consumption of energy rich diets, low in fiber diet; obesity and decreased physical activity while the non modifiable risk factors of diabetes include age, ethnicity, Genetic predisposition are also associated with the development of diabetes mellitus (9).

1.1.3 Pathophysiology of Diabetes mellitus

Type I DM is the result of interactions of genetic, environmental, and immunologic factors that ultimately lead to the destruction of the pancreatic beta cells and insulin deficiency. Most results from beta cell loss is a T-cell mediated autoimmune destruction, some have evidence of islet directed autoimmunity the majority of type I diabetes is of the immune mediated nature (12). Type II DM is a complex metabolic disorder with multiple Pathophysiologic abnormalities. Insulin resistance and beta-cell failure represent the core defects. Patients impaired glucose tolerance (IGT) is near maximally insulin resistant and have lost 80% of their beta cell function. Accelerated lipolysis, gastrointestinal tract incretin deficiency or resistance, hyperglucagonemia, and other factors play major roles in development of glucose intolerance in type II DM (13).

1.1.4 Diagnosis of diabetes mellitus

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. But these symptoms have to be supported by DM diagnostic criteria. For decades, the diagnosis of diabetes has been based on glucose criteria, either the fasting plasma glucose (FPG) or the 75g oral glucose tolerance test (OGTT)(1),which is fasting plasma glucose level at or above 126 mg/dL, plasma glucose at or above 200 mg/dL two hours after a 75 g oral glucose load in a glucose tolerance test and random plasma glucose at or above 200 mg/dL. Epidemiological studies confirmed the long-standing diagnostic 2-h plasma glucose (PG) value of $\geq$ 200 mg/dL. However, the older FPG diagnostic cut point of 140 mg/dL was noted to identify far in fewer individuals with diabetes than the 2-h PG cut point, so $\geq$ 126 mg/dL is now used. Glycated hemoglobin (A1C) 5.7–6.4% is also used commonly to diagnose diabetes in individuals with risk factors (1, 9, 14, 15).

1.1.5 Management and Prevention of Diabetes mellitus

DM is a chronic disease, therefore non pharmacological (life style modification & diet control) and pharmacological approaches (insulin and oral hypoglycemic agents) are required to improve the prognosis of patients, their quality of life and to decrease morbidity and mortality from micro- and macro-vascular complications (16). For type I DM the first and remain the primary treatment option is administration of insulin (15, 16). Management of Type II DM starts from the

non-pharmacologic means and then to the oral hypoglycemic agents such as sulphonylureas, biguanides, thiazolidinediones, meglitinides, in cretins, amylinanalogues, alpha-glucosidase inhibitor etc or Combination of these agents with one another or insulin are used according to the stage of the DM (9, 13, 14).

Primary prevention aimed at long-term weight control because obesity is a major risk factor strongly associated with insulin resistance and lifestyle modifications are likely to be most successful in delaying or preventing type II DM (15).

Nowadays medicinal plants also play a pivotal role in prevention, managing DM and related complications due to their secondary metabolites. Herbs are the main source of medicinal agents and a large number of medicinal herbs are found to be efficacious, cheap and safe in preventing diabetes and diabetic complications (10, 14, 17-19).

1.2 Statement of the problem

Since time immemorial, traditional medicinal plants have been used to treat patients with diabetes mellitus especially type II (8, 18). In many countries of the world, ethno pharmacological studies showed a number of plants used for the management of diabetes and some of them have been experimentally evaluated and the active principles were isolated (13). Ethnobotanical information suggests about 800-1200 medicinal plants may possess antidiabetic potential in the world (19, 20). But little is known on the specific modes of action of these plants for treating diabetes although insulin mimetic or secretagogues activity has been proposed for some (21).

Based on the WHO recommendations hypoglycemic agents of plant origin used in traditional medicine are important source for DM management. Plant agents and herbal formulation are usually less toxic and free from side effects than synthetic one (20, 21).

In spite of the introduction of hypoglycemic agents, DM and its complications continue to be a major health problem worldwide in the 21st century (5). In addition with increasing incidence of diabetes mellitus in resource limited countries throughout the world and due to adverse effects of synthetic medicine, as a result of these serious side effects they are not suitable for use during pregnancy (22).

Investigating the safety and efficacy of these plants in animal model could give valuable information in this regard. The public could be benefited from the use of plants if the safety and efficacy is proved at large, since we are the most users of traditional medicine in the world which is about 90% (23). Thus, there is a clear need for development of indigenous, inexpensive botanical sources for antidiabetic crude or purified drugs.

1.3 The Genus Asparagus

Modern taxonomists have placed the genus Asparagus in family Asparagaceae of order Asparagales rather than in Liliaceae. The family Asparagaceae contains two other genera, Myrsiphyllum and Protasparagus. The genus Asparagus includes about 370 species around the world. Many species of the genus *Asparagus* are medicinally very important and novel chemicals have been isolated from roots, stem and cladodes (24).

1.3.1 Botanical Background *Asparagus africanus Lam.*

A.africanus Lam: - belonging to the family *Asparagaceae* is medicinal shrub valued for its medicinal properties. It is widely distributed throughout Africa including Ethiopia, parts of Europe, Asia and Australia (25). *A.africanus Lam*, with a common name African asparagus is a perennial shrub or climber with stems up to 6 meters high growing between 700 and 3800m above sea level (25) [Figure 1].

Figure 1 Picture of *Asparagus africanus Lam. and its root*

1.3.2 Traditional and Pharmacological uses of *Asparagus africanus Lam*

Traditionally *A. africanus* is used to treat various human ailments in Ethiopia and other African countries including Diabetes mellitus. Two anti protozoan compounds, muzanazagenin and lignan have been isolated from its roots of *A.africanus Lam* to treat different protozoan diseases. The extract of this plant exhibit effects in treating: bilharziasis, syphilis, gonorrhoea, lishmaniasis, malaria, inflammation, impotence which were confirmed scientifically (25-28), anti fertility, as birth canal dilator (29).

A study done in Nigeria on its analgesic and antinflammatory effects showed effective as that of Indometacin, in fact in dose dependant manner. Concerning its toxicity profile, the methanolic extract of the roots of *A.africanus* did not cause mortality up to the dose of 5000 mg/kg orally and thus considered to be none toxic (28). In Kenya, it has been used for centuries as a traditional drug (26). Many species of the genus *Asparagus* including *A. officinalis L, A. ascendens* and *A. racemosus* were researched in various parts of the world for their antidiabetic effects and scientifically validated with different proposed mechanisms of actions, with leading of insulin mimetic (2, 30). The use of *A. africanus Lam*, for management of DM is reported ethinobotany in Nigeria (31). *A. africanus* in Ethiopian folk medicine is also used traditionally for management of DM. On the other hand, since many of the genus Asparagus have shown potential antidiabetic actions, this is clue to investigate *A.africanus* to manage diabetes as supported by previous reports that plants with similar genus having similar effects (32).

1.4 Significance of the Study

DM is one of the most challenging health problems in the 21st century and the world's largest emerging metabolic disorder of the endocrine system, afflicting the globe population at the alarming rate. Despite this managing the growing number of disease cases and developing new agents devoid of adverse effect are other challenges, because most of the drugs in current use are seriously limited by both their side effects and the cost of the treatment.

Medicinal plants have been used traditionally for almost all kinds of diseases directly or indirectly since human history including fighting against DM, especially in resource limited countries like Ethiopia. However, most are without scientific proof for safety, efficacy and dose specification. *A.africanus Lam* is among these commonly used medicinal plants with no scientific validation for Diabetes mellitus. Therefore it needs scientific justification to be validated as safe and effective to combat DM.

It is then an urgent need to search for new antidiabetic drugs which ideally should be not only more efficacious and less toxic than the current ones, but also cheap and available especially in rural areas, thus discovering new agents to overcome these all challenges is straight forward hoping to come up with suitable drug for mankind. It is also important as scientific evidence for promoting our traditional medicine for the rest of the world.

The results of pharmacological screening of claimed plant could have a contribution in the discovery of new medicinal plant with antidiabetic activity. This delivers baseline data for those researchers who are engaged to search medicinal plants with antidiabetic activity. Therefore the present study aims to investigate the antidiabetic activity of root extract of *A. africanus* in rodent models.

2. OBJECTIVES

2.1 General Objective

To investigate the oral antidiabetic activity of crude extract of root of *Asparagus africanus* Lam. (Asparagaceae) in Rodent models.

2.2 Specific Objectives

- To perform preliminary phytochemical screening of the crude root extract
- To assess the acute oral toxicity of the 80% methanolic root extract of *A. africanus*
- To assess hypoglycemic effect of root extract of *A. africanus* in normal fasted mice
- To evaluate effect of root extract of *A. africanus* on Streptozocin induced diabetic mice
- To assess the effect of root extract on oral glucose tolerance test in Glucose loaded rats

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Drugs, chemicals and equipments

The following chemicals, reagents and drug were used for the experimental study: Streptozocin (Sigma Aldrich, China), Glucose standard strip/test kits and one touch glucometer (Accu-check Active®, Germany), Glibenclamide (Cadilla, India), tri-sodium citrate, citric acid and absolute methanol 99.8% (NATASO, India), Distilled water, glucose 40% solution (JE IL Pharma. Co., Daegu Korea), hydrochloric acid and benzene (Nice, Cochin-India), acetic acid, Mercuric chloride, iodine and potassium iodide (Supertek international Pvt, India), concentrated sulfuric acid, chloroform and glacial acetic acid (Avantor performance materials international Pvt,USA), copper sulphate, ammonia and ferric chloride (Avishker international Pvt, India),sodium carbonate (Guangzhou Jinhuada chemical reagent Co.,Ltd, China). All chemicals were analytical grade.

In addition, the following equipments and supplies including : PH213 Microprocessor PH meter (HANNA Instruments, Italy), Whatman filter paper (Whatman LTD, England), mortar and pestle, diabetic syringe, syringe with needle of 3,5 and 10ml, beakers, separating funnels, Aluminum foil, measuring cylinders, Disposable glove, rat and mouse gavages and cages, tea filter, dry oven (250 $\pm$10% Volts AC 600 watts 50/60Hz, France), Electrical balance (Adam equipment CO. LTD), pasture pipette, stainless scissor, Erlenmeyer flask of different sizes were used.

3.1.2. Plant materials

Enough amount of the root of *A. africanus* Lam. was collected in December, 2013 in Amhara region, North Gondar, from University of Gondar Maraki Campus 730 km away from the capital city Addis Ababa. Identification and authentication of the plant specimen was done at Addis Ababa University the Department of Biology Herbarium and plant specimen was kept there with voucher number KA1783.

3.1.3. Experimental animals

Healthy male *Swiss albino* mice (weighing 20–25 g and age of 8–10 weeks) and either sex *Wistar rats* (weighing 150-200 g and age of 2-3 months) were obtained from animal house of Pharmacology Department, School of Pharmacy, University of Gondar, both of them were in breed. Rodents were kept under standard condition (12 h light: 12 h dark cycle; 19-25°C) and pellet diet and water *ad libtum*.

3.2 Methods

3.2.1 Plant material preparation and extraction

The collected fresh roots of *A. africanus* was cleaned with tap water to remove dirt, soil then with distilled water and air dried under shade at room temperature. The dried root was coarsely powdered by using portal and pestle. Two hundred fifty gram was macerated in 80% methanol by using Erlenmeyer flask for 72 hrs at room temperature. After 72 hrs, filtrate was separated from the marc using tea filter and further filtered by Whatman filter paper No.1. The marc was remacerated (33) twice using the same solvent. Following exhaustive extraction, the hydroalcoholic solvent was removed by evaporation in oven under 40°C to obtain the crude extract. The extract was kept in desiccators by appropriate small bottle container until used for experiment.

3.2.2 Preliminary phytochemical screening

Using standard procedures and reagents the crude extract was screened for the presence of secondary metabolites such as flavonoids, terpenoids, saponins, alkaloids, tannins, polyphenolic compounds, cardiac glycosides, reducing sugar, sterols and anthraquinones (33-35) (See annex).

3.2.3 Acute oral toxicity test

Acute oral toxicity for *A.africanus* Lam. extract was determined based on the limit test recommendations of OECD 425 Guideline. On day one, two female Swiss albino mice were fasted for 3-4 h but water was allowed and dosed 2000 mg/kg and 5000 mg/kg for each mouse the extract orally. The mice were then kept under strict observation during the first 4 h for

physical or behavioral changes such as feeding behavior, hair erection, lacrimation, salivation, mortality and other signs of toxicity manifestations for 24 h in different cadges (36).
Following the results from each mouse, other six female mice were randomly selected and fasted for 3-4 h and a single dose of 2000 mg/kg for four and 5000 mg/kg for two mice were administered orally then observed in different cadges in the same manner as above. This observation was continued for further 14 days for any signs of overt toxicity. Based on the result acute toxicity LD50 was calculated for the proceeding procedure (36).

3.3 Pharmacological screening

3.3.1 Grouping and dosing of Rodents

In all models, rodents were divided in to five groups each comprising of six. Hypoglycemic and anti diabetic studies were conducted on healthy male mice (37) in which diabetes was induced using Streptozocin 150 mg/kg with its about 66.7% efficacy of inducing type II diabetes (38, 39). Whereas, for oral glucose tolerance test (OGTT), rats were used since they are preferable in such studies (40, 41). The doses were determined based on the acute toxicity study results of the lower limit test for the subsequent tests, thus the middle dose was one tenth of the limit dose that was 2000 mg/kg, which would be 200 mg/kg, the lower dose was calculated by taking half of middle dose, that means 100 mg/kg and higher dose was calculated as twice the middle dose that was 400 mg/ kg (3).

Then rodents were grouped randomly in to five equal groups (n= 6/group). In all models, group I received distilled water and served as negative control; Group II received a standard, glibenclamide 5 mg/kg; Group III-V received 100, 200 and 400 mg/kg/day of *A. africanus* extract calculated from the results of acute toxicity test result respectively. Each dose of the extract and the control was administered orally for the test rodents (42). Measurement of BGL for each group at each interval of time was done in triplicate and the average value was taken to assure the data quality of the experimental study.

3.3.2 Assessment of the effects of the extract on blood glucose level of normal fasted mice

The mice were fasted overnight for 5-6 h, but water was allowed. Using aseptic precautions, blood was collected by cutting their tip of tails to determine pretreatment blood sugar levels

(BGL) (37, 43). Immediately afterwards, these mice were administered the vehicle, positive control and 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day of the extract according to their respective randomly assigned groups. Blood samples were collected from overnight fasted rodents' tails at 0, 1, 2, 3 and 4 h post treatments for the determination of BGL.

3.3.3 Evaluation of the effect of the extract on Streptozocin induced diabetic mice

Diabetes was induced in mice by a single intraperitonially injection 0.1 M Streptozocin (STZ). 0.1 M citrate buffer was prepared by dissolving 2.1 g of citric acid and 2.94 g of sodium tri citrate in 100 ml of distilled water. The pH was adjusted to 4.5 by the proper addition in drop wise of 0.1 M NaOH/HCL of each using a calibrated pH meter (44). Immediately afterwards, the prepared 0.1 M 150 mg/Kg STZ was injected intraperitonially to the mice fasted 5-6 h hrs with aseptic precautions (37). Seventy two hours later, mice showing blood glucose level >200 mg/dL was selected. These mice were assigned randomly to five equal groups (n=6/group) and treated in the following manner; Group I distilled water, Group II received a standard, glibenclamide 5 mg/kg and Group III-V received 100,200 and 400 mg/kg/day extract respectively. Then BGL was determined at 0, 1, 2, 3 and 4 h post treatment.

3.3.4 Evaluation of the effect of the extracts on oral glucose tolerance test in rats

Rats were fasted for 12-14 h over night (37) and assigned randomly into 5 equal groups (n= 6/group). These rats were orally treated as follows: Group I was received distilled water, Group II received a standard drug, glibenclamide 5mg/kg, Group III-V were received *Asparagus africanus* extract in the same manner as above. One hour later, all these rats were orally loaded with 2 gm/kg glucose solution (45). Blood samples were collected from the tails of these rats immediately prior to commencement of treatment and at 30, 60 and 120 min after the glucose challenge and BGL was determined for each group (37).

3.4 Statistical Analysis

All the grouped data were statistically evaluated and the significance of various treatments was calculated using the one-way analysis of variance (ANOVA) followed by Dunnet's post-hoc test to compare results between and within groups for difference between initial and final results. All the results were expressed as mean ± S*EM* of the six mice and rats in each group. The level of

significance was considered as significant P-value ≤ 0.05. All data processes were done by using SPSS data analysis soft ware of version 20.0.

3.5 Ethical Clearance

Ethical clearance was obtained from Pharmacology Department, College of Medicine and Health Sciences, University of Gondar, before the actual work begun. Rodents were handled according to OECD 425 and Guideline for reporting experiments involving animals: the arrive guideline (36, 46).

4. RESULTS

4.1 Extraction

The percentage yield of 80% methanolic extract of the dried root of *A. africanus* was found to be 42.4% (w/w). The extract was brown, gummy semisolid at room temperature and solidified when stored in desiccators, since the extract was hygroscopic in its nature. It is very soluble in water forming a clear solution.

4.2.Phytochemical test

Preliminary phytochemical screening of the crude extract of *A. africanus* revealed the presence of bioactive secondary plant metabolites such as Flavonoids, alkaloids, tannins, saponins and others (Table 1).

Table 1: Phytochemical screening result of hydroalcoholic root extract of *A. africanus*

Test for secondary metabolite	Observed Results
Flavonoids	+
Terpenoids	+
Saponins	+
Alkaloids	+
Tannins	+
Sterols	+
polyphenolic compounds	+
Cardiac glycosides	-
Anthraquinones	-
Reducing sugars	+

\+ Present - Absent

4.3 Acute oral toxicity test

Acute toxicity study of the methanolic root extract of *A. africanus* did not reveal any behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea and lacrimation. Besides, the extract did not cause mortality in the

mice at a dose of 2000 mg/kg and 5000 mg/kg during the observation time. Thus, the median lethal dose (LD50) of the root extract of the plant is said to be greater than 5000 mg/kg, indicating a very wide safety margin of the extract.

4.4 The effects of root extract of *A. asparagus* on normal fasted mice

As shown in Table 2, the effect root extract of *A. africanus* BGL of normal fasted mice between groups analysis revealed a significant difference among groups when compared with the base line. The plant extract at 100 mg/kg/day failed to show significant hypoglycemic effect at all time points although percent reduction was (23.88%) at the 4^{th} h. However, significant hypoglycemia was recorded for *A. africanus* 200 mg/kg/day at the 3^{rd} ($p<0.05$) and 4^{th} h ($p<0.01$) with (34.16%) and (36.53%) reduction in BGL post treatment compared to the base line, respectively. Glibenclamide 5 mg (GL5) showed lowering BGL as early as the 1^{st} h and lasts till the end of the experiment with ($p<0.001$), when compared with the vehicle. GL5 percent reduction was found to be, 36.55%, 35.20%, 38.83% and 37.82% from 1^{st} -4^{th} h respectively. On the other hand, *A. africanus* 400 mg/kg/day did not cause any hypoglycemia at all time points which were similar to that of the negative control.

Apparent statistical differences were not observed when the different doses of the extract were compared with each other. However, when the standard was compared with different doses of the extract there was statistical significance both with AA100 mg/kg/day and AA400 mg/kg/day from the 1^{st}-3^{rd} h ($p< 0.05$).

Within group analysis revealed that vehicle treated animals did not show any significant reduction in BGL across all time points compared to initial fasting or baseline level and the same is true for both AA200 mg/kg/day and AA400 mg/kg/day. But further within group comparisons for AA100 mg/kg/day extract indicated that there was a fall in BGL at the 3^{rd} and 4^{th} compared to 0 h ($p< 0.05$ and $p< 0.01$ h) respectively.

Table 2: Hypoglycemic effects of 80% methanolic root extract of Asparagus africanus on blood glucose levels in fasted normal mice

	Blood Glucose level in mg/dL				
Group	0 h	1 h	2 h	3 h	4 h
Dis. H_2O	96.00±2.44	89.33±2.82	88.28±3.95	86.34±3.90	84.95±4.91
GL5mg	91.14±4.9	57.83±2.42^{a3b1d3e3}	59.06±3.47^{a3b1d1e3}	55.75±2.5^{a3b3d1e3}	56.67±3.47^{a3b3d3e3}
AA 100mg	95.61±3.76	86.06±5.67	77.39±3.71^{e1}	74.22±3.89^{e2}	72.78±3.12^{e2}
AA 200mg	104.80±11.	81.50±9.42	77.75±7.16	68.78±4.72^{a1}	66.72±3.38^{a2}
AA 400mg	83.50±2.22	83.44±2.73	78.00±3.43	74.83±3.96	73.17±3.54

Values are mean±SEM. n=6; ^{a}compared to Dist H_2O; ^{b}compared to AA100 mg; ^{c}compared to AA200 mg; ^{d}compared to AA400 mg; ^{e}compared to 0h; $^{1}p<0.05$; $^{2}p<0.01$; $^{3}p<0.001$. (Distilled H_2O=negative control; GL 5 mg, glibenclamide 5 mg/kg; AA100, AA200 & AA400= *A.africanus* extracts mg /kg/day).

4.5 The effects root extract of *A. asparagus* on Streptozocin induced diabetic mice

Forty five mice were injected 150 mg/kg Streptozotocin intraperitonially and 34 of them were found to be diabetic, with a success rate of 75.6%. Out of the thirty four mice, one died before the commencement of the experimental procedure, two with non recordable (high) BGL, as a result these mice were excluded but all the rest survived until the end of the experiment.

The effect of root extract of *A. africanus* in Streptozotocin-induced diabetes was depicted in Table 3. Inter-group analysis indicated that no detectable changes were observed for the fasting base line BGL of diabetic control group. Interestingly, mice treated with different doses of extract and glibenclamide revealed fall in BGL compared to their initial values. Accordingly, AA100 mg/kg/day reduced BGL by 22.72% at the 3rd h although it does not reached to statistical significant level but at 4th h ($p<0.01$). However, extracts AA200 mg/kg/day (31.39, 41.09, 56.02%) and AA400 mg/kg/day (23.58, 26.80, 40.85%) since the 2nd h respectively reduced BGL with different significance level except at the 4th h ($p<0.001$ in both cases) compared to vehicle took diabetic control group mice. In the same manner, GL5 ($p<0.01$, $p<0.001$ and $p<0.001$) did show a significant BGL reduction at the 2nd, 3rd and 4th h consecutively. Maximum BGL lowering effects were attained at the 4th h, with percent reduction of 38.62%, 56.02%, 40.85%, and 58.66% for AA100 mg/kg/day, AA200 mg/kg/day, AA400 mg/kg/day and GL5 mg/kg/day respectively. No significant detectable changes were observed in BGL when, either

different doses of the extract compared to each other as well as when all extract doses compared with the positive control at all time points in cases of between group analysis of diabetic model.

Intra-group analysis demonstrated that vehicle treated mice had no effect on BGL at all-time points compared to the base line. Subsequent within group analysis indicated that AA100 mg/kg/day do not significantly decreased BGL at all-time points. On the other hand it is worse nothing that AA200 mg and GL5 mg significantly controlled BGL in a similar pattern, with similar p-values becoming very significant at the 4th h ($p<0.001$ in both cases).

Table 3: Antidiabetic effects of 80% methanolic root extract of *A. africanus* on blood glucose level in Streptozotocin induced Diabetic mice

	Blood glucose level in mg/dL				
Group	0 h	1 h	2 hrs	3 hrs	4hrs
Distilled H_2O	347.61±41.82	347.84±37.70	341.33±15.44	337.22±11.69	332.17±11.13
GL 5 mg	337.22±33.32	304.94±36.03	217.33±17.11^{a1e1}	177.67±13.19^{a3e1f1}	139.39±12.00^{a3e2f2}
AA100 mg	340.17±39.26	330.17±37.54	277.39±39.45	262.89±34.69	208.78±42.19^{a2}
AA 200 mg	339.78±36.23	317.89±30.62	233.11±10.60^{a1e1}	200.17±14.92^{a2e1f1}	149.44±13.33^{a3e1f1}
AA 400 mg	287.95±31.50	260.56±33.10	220.06±32.70^{a2}	210.78±27.58^{a1}	170.33±32.09^{a3}

Values are mean±SEM. n=6; ^{a}compared to Dist H_2O; ^{b}compared to100 mg; ^{c}compared to 200 mg; ^{d}compared to 400 mg; ^{e}compared to 0h; ^{f}compared to 1h; $^{1}p<0.05$; $^{2}p<0.01$; $^{3}p<0.001$. (Distilled H_2O=negative control; GL 5 mg, glibenclamide 5 mg/kg; AA100, AA200 & AA400= *A.africanus* extracts mg /kg/day).

4.6 The effects of *A. asparagus* extract on oral glucose tolerance test in glucose loaded rats

Effects of the extract of *A. africanus* OGTT were shown in Table 4. BGL of all groups prior to extract administration (0 min) showed no apparent difference compared to each other. However all groups, revealed rapid increase in BGL by 32.0 to 42.13% from initial level, 30 min following extract administration or after one hour oral glucose loading, confirming the induction of hyperglycemia. Hyperglycemia with glucose challenge was not significantly brought down with the distilled water handled rats at all time points. AA100 mg/kg/day produced a detectable BGL changes at 60 min and 120 min ($p<0.01$, $p<0.001$) with 32.28% and 36.95% utilized glucose consecutively. In the same way AA200 mg/kg/day caused a very significant reduction of BGL at 60 min and 120 min ($p< 0.001$ in both cases) with percentage of 34.01%, 43.48% respectively.

A.africanus 400 mg/kg/day extract caused a fall in BGL to a significant level only at 60 min ($p<0.01$ with 34.36%) but at 120 min although percentage of BGL reduction was more than double compared to the vehicle which was 29.97%, found to be non significant statistically. GL5 produced significant improvement of hyperglycemia ($p<0.001$) both at 60 and 120 min with maximum BGL at the 4^{th} h, 46.32%.

Further, inter group analysis of OGTT revealed there was statistical significance in BGL utilization among different doses of extracts in particular AA200 mg/kg/day both with AA100 mg/kg/day and AA400 mg/kg/day at 120 min ($p<0.05$, $p<0.001$) respectively and the same analogy was true with the standard. There was also statistical differences AA100 mg/kg/day in comparison with AA400 mg/kg/day at 120 min. In the same manner as the above models here also AA200 mg/kg/day and GL5 mg/kg/day compete to each other in utilizing glucose.

Intra-group analysis revealed that there was statistical significance beginning from 60 min onwards compared to 30 min for AA100 mg/kg/day, AA200 mg/kg/day and the standard but not for the vehicle and the higher dose.

Table 4: The Effects of 80% methanolic root extract of *Asparagus africanus* on Oral Glucose Tolerance Test in rats

	Blood glucose level in mg/dL			
Group	0min	30min	60min	120min
Dist H_2O	84.45±2.23	134.33±1.89	125.61±1.36	116.11±2.42
GL5 mg	84.95±2.83	125.22±3.71	81.33±4.69^{a3e3}	67.22±2.51^{a3b3d3e3}
AA100 mg	85.56±2.49	147.84±6.11	100.11±5.03^{a2e1}	93.22±1.51^{a3d2e3}
AA 200 mg	85.72±1.04	140.67±5.84	92.83±2.96^{a3e2}	78.00±6.90^{a3b2d3e3}
AA 400 mg	93.17±7.53	155.89±6.74	102.33±9.44^{a1e1}	109.17±3.78

Values are mean±SEM. n=6; [a]compared to Dist H_2O; [b]compared to100 mg; [c]compared to 200 mg; [d]compared to 400mg; [e]compared to 30min; [1]$p<0.05$; [2]$p<0.01$; [3]$p<0.001$. (Distilled H_2O, negative control; GL 5 mg, glibenclamide; 5 mg/kg; AA100, AA200 & AA400= *A.africanus* extracts mg /kg/day).

5. DISCUSSION

Currently management of Diabetes Mellitus and its complications with minimal side effect agents is the major challenge to the medical care system (47). Hence this leads to extensive investigation of efficacious and potent natural antidiabetic products with fewer side effects of plant origin. The current study on *A. asparagus* is among primarily focuses on plants since they are source of secondary metabolites which are implicated in amelioration of DM and related complications with the hope to come up with less side effects or non toxic antidiabetic agent.

Streptozotocin (STZ) is by far the most frequently used diabetogenic agent (69%) in the preparation of diabetic animal models for the investigation of multiple aspects of diabetes (38). It is DNA alkylating agent which is glucose analogue, cytotoxic to pancreatic β-cells and its effects can be seen within seventy two hours after administration depending on the dose administered. In fact the dose required for inducing diabetes depends on the animal species, route of administration and nutritional status of the animals (48, 49). Partially destructed beta cells by STZ resulted in insufficient insulin secretion causing type II diabetes which is very important for the advancement of our knowledge and understanding of the various aspects of its pathogenesis and ultimately finding new therapies. Thus STZ induced animal model of diabetes is widely accepted and reported to resemble that of human hyperglycemic non ketotic diabetes mellitus (32, 43). As a result, this model was selected in the present study.

The acute oral toxicity test of *A. africanus* extract in mice produced no sign of toxicity or death even at the dose of 5000 mg/kg which shows very safe, well tolerated and non toxic in the tested rodents which is similar to studies done earlier (27, 28), though direct extrapolation to human is difficult due to inter species variation. The test doses are determined based on the acute toxicity study of the limit test of 2000 mg/kg which is the lower dose level calculated by taking half of the middle dose, that would be 100 mg/kg and the middle dose is one tenth of the limit dose which is 200 mg/kg. Higher dose is calculated as twice the middle dose becomes 400 mg/kg (3). Even though the extract is non toxic up to 5000 mg/kg the lower limit dose is selected for the test doses because most literatures stated that extracts have no as such practical antidiabetic activity above 200 mg/kg extract (32, 42, 50, 51), in which this study also goes in line with.

The assessment of the glucose handling in experimental rodents of *A.africanus* root extract is successfully achieved by determination of secondary metabolites through phytochemical screening using standard procedures which are positive for flavonoids, terpenoids, phenols, saponins, alkaloids and others (Table1). They are potential bioactive molecules of this plant which are responsible for ameliorating chronic hyperglycemia as their potential effects are confirmed in normal fasted, STZ induced diabetic mice and glucose utilizing capacity in postprandial rats.

The study has shown that no detectable changes observed in baseline BGL across groups in both normal (Table 2) as well as diabetic mice (Table 3), however, significant BGL reductions started to appear after the hydroalcoholic extract and the standard drug are administered, indicating that changes produced are attributed to treatments received. Therefore, results of this study obviously indicates that the extract reduces BGL level in normal, in fact AA200 mg only, and diabetic mice for all different doses of extract plus in glucose induced hyperglycemic rats.

Among the various doses of the extract, maximum activity is achieved with AA200 mg in all test models. It is great success this dose is capable of bringing down Streptozocin-induced hyperglycemia close to the standard drug values (Table 3). This dose of extract also brought the hyperglycemic state in OGTT down within 60 min ($p<0.01$) and with the same level of significance ($p<0.001$) at 120 min in the same pattern with that of GL5 (Table 4). This oral glucose tolerance test also confirmed blood glucose lowering activity of *A. africanus* extract in steady state manner. Thus, it is possible to speculate that the extract and glibenclamide might produce hypoglycemic, antidiabetic and enhancing of glucose utilization effects by similar mechanisms in diabetes that means by enhancing insulin release or insulin-like effect. Because its genus and other numerous plants extract have been reported to have similar mode of action with that of glibenclamide, providing evidence to this similar insulin enhancing to utilize glucose and insulin releasing effects of the root extract (2, 21).

It was observed that the extract exerted its action in a non-dose dependent but time dependant manner; because the higher dose produced less activity and maximum activities achieved in all models in the last hours except AA400 mg in OGTT model. This could be the active principles in the extract need time to reach sufficient concentration at the target site or in the higher doses there could be other interfering substances those preclude the expected higher hypoglycemic

effect, as a similar pattern was reported with its many species of the genus Asparagus, in fact for some with dose dependent actions, and other plants (2, 39, 52, 53).

The lower dose, AA100 mg of the extract appeared to be ineffective in reducing BGL in normal mice (Table 2). This could be attributed to inability of the dose to overcome counter-regulatory physiological mechanisms of the lesser concentration active principles to induce hypoglycemia or the small sample size employed that prohibited statistical significance. AA400 mg also failed to lower BGL at all time points in normal mice, this is probably due to the presence of other interfering substances with the active principles at the higher doses (32) or the extract is having reducing sugar so the mice may be utilized it. This observation suggests that activity might decrease with dose, because BGL reduction is, most likely, the synergistic effects among various constituents of the extract (32, 39).

On the other hand, AA100 mg produced antidiabetic action in Streptozocin-induced mice, which might imply that the hypoglycemic nature of the lower dose would be apparent when there is an alteration in normal blood glucose regulatory mechanisms in diabetes. This could be explained by; during fasting time the body stimulates the release of the hormone glucagon, which in turn releases glucose into the blood through catabolic processes. Normally, the body produces and processes insulin to counteract the rise in glucose levels but in diabetes, this process is impaired (11), so this blood glucose lowering effect in altered pancreas is most likely as a result of antidiabetic activity of the extract via its different mechanisms action achieved in of diabetic mice with lower dose, that may relief oxidative stress, regeneration of β-cells which lost as a result of cytotoxic effects of STZ. On the other hand, this is why the AA100 mg might be failed to induce hypoglycemia in normal mice but in diabetic once.

Interestingly, in OGTT the extract showed significant reduction in BGL from 60 min on-wards except for the higher dose. This suggests that the extract has a potential to enhance glucose utilizing capacity via different mechanisms, reflecting a potential advantage of the extract in minimizing hyperglycemia which is a prerequisite for diabetes related complications (39, 51). But in case of the higher dose the ability of the extract fall to utilize blood glucose as time goes on in OGTT model, this is probably due to high glycaemic index of the extract in which both in normoglycemic and OGTT tend to raise in BGL in case of higher dose, as supported by other studies (39).

The overall hypoglycemic, antidiabetic and glucose utilizing potential of the extract is attributed to its bioactive constituents because they attenuate diabetes and diabetic complications through different mechanisms of actions (54).

Numerous mechanisms of plants extract actions are proposed: some hypothesis relates to their effects on the activity of pancreatic beta cells (insulin release), by inhibiting effect of insulinase, increase insulin sensitivity or the insulin-like activity of the plant extracts, increase in peripheral utilization of glucose, increase of synthesis of hepatic glycogen or decrease glycogenolysis, inhibition of intestinal glucose absorption, reduction of glycaemic index of carbohydrates and others (21).

A number of experimental and clinical studies have indicated that hyperglycemia may directly or indirectly contribute to an increased formation of free radicals which has been implicated in diabetic and related complications, consequently to the onset of oxidative stress. Oxidative stress is a condition of reduction in antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase and Catalase levels. Thus reducing this oxidative stress is another main target for bioactive plant constituents (3, 55-57); here is more discussion for most of them, which are identified in root extract of *A.africanus*.

Flavonoids: are known for their health promoting properties due to their high antioxidant capacity and have been described to be excellent free radical scavenging agents. They are the most widespread polyphenolic substances with hypoglycemic and antidiabetic properties constituting active biological principles of most medicinal plants, in which phytochemical testing also proved its presence in the studied plant. They mainly act by inhibiting free radical formation and propagation of free radical reactions through hydrogen donation and aromatic hydroxylation. They reduce oxidative stress leading to less degradation of GSH (glutathione) or either increases the biosynthesis of GSH and also leads to the regeneration of pancreatic β-cells, reduces necrosis and degeneration, thus may be effective in treating hyperglycemia thereby preventing diabetes and its complications as found in extract of *A.africanus* (16, 51).

Alkaloids: since the extract was positive for alkaloids which are implicated in producing anti hyperglycemic action by potentiating pancreatic secretion of insulin from β-cell of islets or by enhancing transport of blood glucose to peripheral tissue are able to restore the reduced GSH

content in diabetic liver which play an important role in prevention of diabetic complications, in addition can modulates enzymes responsible for glucose metabolism, reducing oxidative stress and thus helps in restoring antioxidant status (58).

Phenolic constituents: The scavenging ability of the phenolic is mainly due to the presence of hydroxyl groups. Being a potent radical scavenger it inhibits the free radical mediated formation of advanced glycation end products and thus are beneficial for counteracting hyperglycemia and complications associated with diabetes (49, 54). Moreover, enhanced insulin secretion by regeneration of β-cells reduces oxidative stress and modulates enzymes responsible for glucose metabolism. (59). Plants rich in phenolic content as *A.africanus* have been reported to possess higher antioxidant activities even than vitamins and synthetic antioxidants, indicating their ability to reduce blood glucose concentration and subsequent oxidation (56, 60) so the extract most likely does some of these actions.

Terpenoids: stimulate release of insulin from pancreas and ameliorates oxidative stress, thus can be effective in management of diabetes and related complications (59). Saponins and tannins are also attributes to observed hypoglycemic effects of *A.africanus* extract (56). Not only these but also other miscellaneous constituents of the plant extract in which we did not test might also speculated to attenuate diabetes and its complications by different mechanisms of modulation of pancreas or extra pancreatic effects as most are already mentioned.

Furthermore, chemical constituents of *A.africanus* root extract are similar with previously done studies on the different species of *Asparagus,* which is clue for speculation for the same mechanism of actions. For instance, the root extract of *A. racemosus (2)* increased insulin secretion in the isolated islets of rats in vitro (61) and *A. officinalis* treated diabetic rats showed antidiabetic effects. The *A. officinalis* treated diabetic rats again showed improvement in the size of the islets of the pancreas (52). Thus the overall anti diabetic effects of the root extract of *A.africanus* is most likely due to the enhanced insulin secretion from the pancreatic β-cells in tested rodents in similar fashion as those of its species. The clinical trial done on *A. officinalis* augments the confirmation of similar biological effects in terms of glucose metabolism through insulin secretion of the plant extract (62).

The major probable mechanisms of the extract that causes insulin secretion are: suppression of oxidative stress, increasing viability and proliferation of β-cells, through increasing ATP/ADP ratio and intracellular Ca^{2+} concentration and others by combined synergistic actions (47) because the plant extract possesses numerous bioactive constituents for the implicated actions.

Therefore from numerous evidences suggested above the mechanisms of BGL lowering actions of hydroalcoholic extract in normal fasted, diabetic mice and glucose loaded rats are most likely due to increase in insulin level in plasma by increasing either the pancreatic secretion of insulin from existing beta cells or in the long run by regenerating pancreatic beta cells subsequently secretion of insulin, its release from the bound form, antioxidant activity or exert its antidiabetic actions through extra-pancreatic, most likely by synergism or individually of the detected secondary metabolites of root extract in tested rodents.

Thus this study clearly indicated that the root extract of *A.africanus Lam*. could be considered as a potential candidate for management of diabetes, through different proposed mechanism of actions either modulating the pancreas, particularly by insulin releasing or extra-pancreas effects supporting the traditional claim.

6. CONCLUSION

Phytochemical screening of *A.africanus* demonstrated that the root extract constitute the major bioactive potential molecules which are implicated in amelioration of diabetes and related complications. The root extract has a very wide safety margins in tested mice which was non toxic up to the dose of 5000 mg/kg. The plant extract showed antidiabetic activity in all employed rodent models, in non-dose dependant but time dependant manner. Therefore, this study clearly indicated that the extract could be considered as a potential candidate for the management of diabetes.

However, further investigation is required for its effects on antioxidant activity, isolation and characterization of the antidiabetic bioactive compound and the establishment of the exact mechanism(s) of action. Thus, this study supports the traditional use of the root of *A. africanus* with a very wide safety margin on studied rodents, even though direct extrapolation to human is inconclusive, for the management of diabetes mellitus.

7. RECOMMENDATIONS

Based on the present study the following recommendations are proposed:

- ✓ Conservation of this precious medicinal plant
- ✓ Investigation of its antioxidant and lipid profile
- ✓ Beside the roots, other parts might require investigations of its antidiabetic activity
- ✓ Fractionation, isolation and characterization of antidiabetic bioactive compound and the establishment of the exact mechanism(s) of action and
- ✓ Further toxicological studies including chronic toxicity

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ANNEX

1. Phytochemical Screening Test (Qualitative Method)

1.Test for alkaloids: Weigh 1g of methanolic dry extract and dissolve 10ml of acid alcohol and boiled for 20minutes at 100 °C in a water bath and filter using Whatman filter paper, take 5ml of the filtrate, add 2ml 10% ammonia, 5ml chloroform and shake gently to extract the alkaloid base. Extract the chloroform layer with 10ml of 70 % acetic acid and divide the solution in two portions in a test tube

A. Few drops of Mayer's reagent ($HgCl_3$ and KI solution) was added to one portion, creamy precipitate was observed indicating the presence of alkaloids

B. Wagner's test: Few drops of Wagner's reagent (Prepared solution of 1.27 gm Iodine and 2 gm KI in 100 ml distilled water) 2-3 ml added to the test sample; reddish brown color was observed confirming the presence of alkaloids.

2.Test for Flavonoids: Weigh 0.2g of extract and 10ml of ethylacetate was added heated on a water bath for 3 minutes. The solution was cooled, filtered then tested for the presence of flavonoids. A. Ammonium test: On 3ml of filtrate 1ml of diluted (10%) ammonium solution was added and then shaken. The yellow color observed in the ammonia layer indicating the presence of the flavonoids

B. Aluminum chloride solution (1% test): 3ml of the filtered solution was shaken with 1ml of 1% aluminum chloride solution. The separated yellow color was formed on the aluminum chloride confirming indicates the presence of flavonoids.

3.Test for saponins: weigh One gram of extract dry powder and dissolve in 5ml distilled water, boiling for 20 minutes at 100 °C in a water bath and filter using Whatman filter paper and add 3ml distilled water to the filtrate, shake vigorously for about 5 minutes. Frothing which persisted on warming is taken as an evidence for the presence of saponins.

4.Test for tannins: weigh 0.5g of the extract dry powder and dissolve in 10ml of distilled water and boiling for 20 minutes at about 100 °C in a water bath and filter using Whatman filter paper and add 3 drops of 0.1% of ferric chloride to the filtered solution. A brownish green or blue black coloration indicate the presence of tannins.

5. Sterols determination: weigh 0.2g of dry extract powder, dissolve in 10ml chloroform and shake to ensure dissolution, add 2ml of 70% acetic acid to the solution cool in ice in

refrigerator for 15 minutes and add 2ml of concentrated sulfuric acid carefully. The color from violet to green in some samples indicates the presence of steroids.

6. Test for cardiac glycosides: weigh 0.5g of dry powder extract and dissolve in 5ml of distilled water, treated with 2 ml of glacial acetic acid, one drop of 0.1% ferric chloride solution added and finally added 1ml of concentrated sulfuric acid. A violet ring appear below the brown ring or a greenish ring form just above the brown ring and gradually spread throughout this layer which confirmed the presence of glycoside.
7. Test for terpenoids: weigh 0.2g of dry extract dissolved in 2ml distilled water to form 10%w/v solution of the extract, add 2ml of chloroform and shake, then add 3ml concentrated sulfuric acid carefully to form a layer. A reddish brown colouration of the interface formation indicates positive results for the presence of terpenoids.
8. Tests for anthraquinones: weigh 1g of dry powder extract, dissolve in 10 ml of benzene shake, filtered with Whatman filter paper, add 5ml of 10% ammonia solution to the filtrate and shake. The presence of a pink, red or violet colour in the ammonical (lower) phase indicates the presence of free anthraquinones.
9. Test for reducing sugar: weigh 1g of dry powder extract; dissolve in 10 ml distilled water then filtered. Filtrate was treated with Benedict's reagent (1gm sodium carbonate, 1.73gm sodium citrate and 0.173gm copper sulfate solution) heated gently for 5 min. Orange red precipitate indicates the presence of reducing sugars.
10. Test for phenols:-Weigh0.2g of dry powder extract, dissolve in 5ml of 95% ethanol then add 2 drops of 1M ferric chloride solution. The presence of intense color indicates the presence of phenols.

2. Preparation buffer solution to adjust Streptozocin (PH =4.5)

Preparation of 0.1 M citrate buffer (pH 4.5)

0.1M citric acid solution.

5.26 g citric acid monohydrate (MW = 210.14 g/mole) was weighed and in dissolved in 250 ml distilled water.

0.1 M tri sodium citrate solution

In the same manner 7.36 g tri sodium citrate dihydrate (MW = 294.12 g/mole) was weighed dissolved in 250 ml distilled water.

For the preparation of 100 ml 0.1 M citrate buffer (pH = 4.5), 47 ml 0.1 M citric acid solution was mixed with 53 ml 0.1 M tri sodium citrate solution to give a 0.1 M citrate buffer solution with pH = 4.5. The solution was kept in refrigerator at 1- 4oC for future use. Then the PH was adjusted to 4.5 by adding 0.1M Hcl and 0.1M NaOH drop wise.

DECLARATION

I hereby declare that this thesis is my bonafide work and that all sources of chemicals, reagents, drugs and materials used for this thesis have been duly acknowledged. The thesis has been submitted in partial fulfillment of the requirements of MSc degree at University of Gondar and deposited at the university's library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Name: Kefyalew Ayalew Signature ----------

Place: University of Gondar, Gondar

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